Regulation of Vascular Proteoglycan Synthesis by Angiotensin II Type 1 and Type 2 Receptors

RYOKO SHIMIZU-HIROTA, HIROYUKI SASAMURA, MIZUO MIFUNE, HIDEAKI NAKAYA, MARI KURODA, MATSUHIKO HAYASHI, and TAKAO SARUTA
Department of Internal Medicine, School of Medicine, Keio University, Tokyo, Japan.

Abstract. Recent studies have shown that proteoglycans play an important role in the development of vascular disease and renal failure. In this study, the effects of angiotensin II (AngII) type 1 (AT1) and type 2 (AT2) receptor stimulation on glycosaminoglycan and proteoglycan core protein synthesis in vascular smooth muscle cells (VSMC) were examined. Treatment of AT1 receptor-expressing VSMC with AngII resulted in a dose-dependent and time-dependent increase (2- to 4-fold) in 3H-glucosamine/35S-sulfate incorporation, which was abolished by pretreatment with the AT1 receptor antagonist, losartan. The effects of AngII were inhibited by the epidermal growth factor receptor inhibitor, AG1478, and the mitogen-activated protein kinase kinase inhibitors, chelerythrine and staurosporine. AngII treatment also resulted in significant increases in the mRNA of the core proteins, versican, biglycan, and perlecan. The effects of AT2 receptor stimulation were examined by retroviral transfection of VSMC with the AT2 receptor. Stimulation of the AT2 receptor in these VSMC-AT2 cells resulted in a significant (1.3-fold) increase in proteoglycan synthesis, which was abolished by the AT2 receptor antagonist, PD123319, and attenuated by pretreatment with pertussis toxin. These results implicate both AT1 and AT2 receptors in the regulation of proteoglycan synthesis and suggest the involvement of epidermal growth factor receptor–dependent tyrosine kinase pathways and Gαi/o-mediated mechanisms in the effects of the two receptors.

Proteoglycans are important nonfibrous components of the extracellular matrix (1). These complex macromolecules consist of a protein core covalently bound to one or more glycosaminoglycan (GAG) side-chains. Because of their structural properties, particularly the strong negative charge carried by the GAG moiety, proteoglycans have many unique functions. These include the ability to bind an array of other molecules, such as growth factors and cytokines, and regulate their function. Consequently, the proteoglycan-rich extracellular matrix serves as both a reservoir and a modulator of the actions of a variety of other growth and differentiation factors. In addition, proteoglycans play a direct role in controlling cell growth and differentiation, in modifying cell adhesion, migration, and development, and in influencing the deposition of other extracellular matrix components such as collagen. The proteoglycans, perlecan and/or agrin, may also play a major role in forming the glomerular basement membrane negative charge barrier, which prevents the loss of protein in the glomerular ultrafiltrate (2,3,4).

Recent studies have shown that abnormal regulation of proteoglycans plays an important role in the pathophysiology of both vascular and renal disease. Abnormal accumulation of proteoglycans has been shown to occur in both atherosclerosis and in postinjury restenosis in both humans and animal models (1,5). It has been proposed that binding of proteoglycans to LDL can cause LDL retention in the artery, which can contribute to the development of atherosclerotic lesions. Two recent reports have also shown that abnormal accumulation of proteoglycans is also a characteristic of a variety of renal diseases in humans, including primary glomerulonephritis, diabetic nephrosclerosis, hypertensive nephrosclerosis, and amyloidosis (6,7). Human studies have also shown that expression of proteoglycans correlates with loss of renal function (8), suggesting an important pathophysiologic role for these glycoproteins in the progression of renal failure.

These reports highlight the importance of understanding the mechanisms of control of proteoglycan synthesis to the design of better strategies for arresting disease development. The proteoglycans were previously classified biochemically into chondroitin sulfate (CSPG), dermatan sulfate (DSPG), and heparan sulfate (HSPG) proteoglycans without a clear knowledge of the structure of their core proteins. However, the use of molecular cloning techniques has revealed the structure of the major core proteins, resulting in a new classification that is based on the core protein moiety. It is now known that major proteoglycan core proteins expressed in the kidney include the large CSPG, versican, the smaller CS/DSPG, decorin and biglycan, and the HSPG, perlecan (2). However, the regulation mechanisms of these proteoglycans are still unclear.

Studies from our laboratory have shown that the vasoactive peptide hormone angiotensin II (AngII) can regulate the synthesis of the extracellular matrix protein collagen via both the type 1 (AT1) and type 2 (AT2) AngII receptors (9,10). We have also shown that treatment with AT1 receptor antagonist
causes proteoglycan core protein-specific changes in hypertensive rats (11). There has recently been a rapid increase in the use of renin-angiotensin inhibitors (angiotensin-converting enzyme inhibitors and AT1 receptor antagonists) to inhibit the progression of renal and cardiovascular disease (12). These two agents differ in that the former reduces stimulation of both AT1 and AT2 receptors, whereas the latter increases AT2 receptor stimulation. The long-term consequences of these differences are still unclear. These two receptors couple to different G proteins, and the AT1 receptor is thought to act predominantly via Gq, whereas the AT2 receptor may act through Goi (13,14). At present, the effects of AT1 and AT2 receptor stimulation on regulation of proteoglycan synthesis have not been defined. The following were, therefore, the aims of this study: (1) to examine the role of the AT1 receptor in the control of proteoglycan synthesis in vascular smooth muscle cells (VSMC) as well as the mechanisms involved; (2) to correlate the observed changes in GAG synthesis to changes in core protein gene expression; and (3) to examine the effects of AT2 receptor stimulation on proteoglycan synthesis.

Materials and Methods
Cell Cultures and Transfection of the AT2 Receptor
Rat VSMC were prepared from the thoracic aortae of 6-wk-old male Wistar rats by collagenase digestion and cultured in Dulbecco’s modified Eagle medium supplemented with 10% fetal calf serum (15). Retroviral transfection of VSMC with an AT2 receptor retrovirus (LXSN-AT2) to produce AT2 receptor-expressing VSMC (VSMC-AT2) was performed as described previously (10).

Proteoglycan Synthesis Assays
Synthesis of cell-associated and medium-secreted proteoglycan was determined as described (16,17) with minor modifications. In brief, quiescent VSMC in 24-well plates were transferred into serum-free media for 48 h. After serum deprivation, cultures were incubated in Dulbecco’s modified Eagle medium containing 3H-glucosamine (2 μCi/ml) or sulfate-free medium containing 35S-Sulfate (5 μCi/ml) in the presence of AngII (10−7 mol/L, unless otherwise stated) for 48 h. VSMC-AT2 cultures were stimulated with the AT2 agonist, CGP42112A (10−7 mol/L, unless otherwise stated). The medium was harvested, and 300 μl of the supernatant was incubated with 25 ml of 2.5% cetylpyridinium chloride (CPC) in the presence of 5 μCi/ml HCl and 80 mM ethylenediaminetetraacetic acid, 0.1 mol/L NaCl, 0.5% Triton X-100. Bound radioactivity was eluted with a NaCl gradient (0.1 to 0.7 mMol/L in the same buffer) and quantified by scintillation counting.

Protein Kinase C (PKC) Assay
Serum-starved VSMC were treated with PKC inhibitors for 30 min, washed twice with cold phosphate-buffered saline, and scraped into extraction buffer (20 mMol/L Tris [pH 7.5], 0.5 mMol ethylene glycol-bis(β-aminoethyl ether)-N,N,N’,N’-tetraacetic acid, 0.5 mMol/L ethylenediaminetetraacetic acid, 0.5% Triton X-100, 25 μg/ml aprotinin, 25 μg/ml leupeptin). PKC activity was assayed by following the method of Luo et al. (19).

Northern Blot Analysis
Total RNA was purified from VSMC and VSMC-AT2 cells by the acid guanidine-phenol-chloroform method and quantified by measurement of absorbance of 260 nm in a spectrophotometer. Twenty micrograms of total RNA were denatured with formamide and formaldehyde at 65°C for 10 min and fractionated by electrophoresis through a 1.0% formaldehyde-agarose gel. RNA was stained with ethidium bromide to verify integrity and equal loading, transferred to a nylon filter (Pall BioSupport, East Hills, NY), and then crosslinked by using a UV irradiator (Stratagene, La Jolla, CA). Prehybridization was conducted at 42°C for 2 h in a buffer containing 6× SSC (0.9 mol/L sodium chloride, 0.09 mol/L sodium citrate [pH 7.0]), 5× Denhardt’s solution (0.1% [wt/vol] polyvinylpyrrolidone, 0.1% [wt/vol] ficoll type 400, 0.1% [wt/vol] bovine serum albumin), 50% formamide, 0.1% sodium dodecyl sulfate, and sheared, denatured salmon sperm DNA (100 μg/ml). The cDNA probe for biglycan (20) was generously provided by Dr. Kevin Drexler (Weis Center for Research, Danville, PA) through Dr. Minoru Takagi (Department of Anatomy, NIH, University School of Medicine, Tokyo, Japan). Probes for perlecan and versican were obtained by reverse transcriptase-PCR (RT-PCR) as described below. Perlecan sense and antisense primers (21) were 5’-GCTGAGGGCTCAGATGGG-3’ and 5’-TGCCCAGGCGTCGGAACT-3’, corresponding to bases 1 to 18 and antisense of 480 to 496 respectively of the previously reported perlecan sequence (RDP-1). Versican sense and antisense primers (22) were 5’-GACTATGGCT-GGCAAA-3’ and 5’-GCTCTTTGGTATGCCAGA-3’, which correspond to sense and antisense respectively, of bases 6813 to 6829 bp and 7371 to 7387 bp in the rat versican sequence. Reaction products were subcloned into the plasmid pCDNA3.1-His (Invitrogen, Carlsbad, CA) and sequenced by using an automated sequencer. The 1.1-kb human GAPDH probe was purchased from Clontech (Palo Alto, CA). Probes were radiolabeled with α-32P dCTP by the random primer synthesis method (RadPrime DNA Labeling System; Life Technologies-BRL, Grand Island, NY). After hybridization, the filter was washed in 0.2× SSC, 0.1% sodium dodecyl sulfate at 42°C. Bands were visualized, and incorporated radioactivity was quantified by scanning with a laser image analyzer (model BAS 2000; Fuji Film, Tokyo, Japan).

Reagents
Cell culture reagents, RT-PCR, and electrophoresis reagents were obtained from Life Technologies-BRL, Perkin Elmer (Branchburg, NJ), and Biorad (Hercules, CA) respectively. Radiochemicals were above. To separate proteoglycans in the media on the basis of differences in charge density, ion-exchange chromatography was performed as described by Kaji et al. (18) using DEAE-Sephacel (Amersham-Pharmaacia, Upsala, Sweden). After application of control and AngII-treated samples, unbound radioactivity was removed from the column by washing with 30 ml of wash buffer (8 mol/L urea, 50 mMol/L Tris [pH 7.5], 2 mMol/L ethylenediaminetetraacetic acid, 0.1 mol/L NaCl, 0.5% Triton X-100). Bound radioactivity was eluted with a NaCl gradient (0.1 to 0.7 mMol/L in the same buffer) and quantified by scintillation counting.
obtained from NEN (Boston, MA). Other chemicals were from Sigma or Calbiochem, unless otherwise stated.

Statistical Analyses

Results are expressed as the mean ± SEM. Statistical comparisons were made by ANOVA followed by Fisher’s post hoc test. Values of $P < 0.05$ were considered statistically significant.

Results

Effects of AT1 Receptor Stimulation on Proteoglycan Synthesis in VSMC

Preliminary experiments established that the VSMC used in this study expressed only the AT1 receptor subtype, whereas the AT2 receptor was not detectable even by RT-PCR assay, as described previously (15,10). When these cells were treated with AngII, a clear increase in proteoglycan synthesis was seen. As shown in Figure 1, AngII stimulation resulted in a time- and dose-dependent increase (2- to 4-fold) in both cell-associated and secreted proteoglycan synthesis. To characterize the proteoglycans biochemically, the supernatants were pretreated with the enzymes Chondroitinase ABC (to detect CSPG and DSPG), Chondroitinase AC (to detect CSPG), and heparitinase (to detect HSPG). As shown in Table 1, increases in Chondroitinase ABC- and AC-sensitive incorporation were readily detectable, suggesting that AngII treatment resulted in increases in all three classes of GAG (CSPG, DSPG, and HSPG). Figure 2 shows the results of ion-exchange chromatography on media from VSMC treated with or without AngII. Two major peaks were eluted by NaCl gradient. The counts from peaks I and II were reduced by heparitinase and Chondroitinase ABC treatment, respectively, suggesting that peak I contained predominantly HSPG whereas peak II contained CS/DSPG, as reported previously (18). Both peaks were increased in the AngII-treated samples, further suggesting that the different classes of GAG were up-regulated by AngII treatment.

Effects of Inhibitors on AT1 Receptor–Induced Increases in Proteoglycan Synthesis in VSMC

To confirm that the above effects were mediated via the AT1 receptor, experiments were performed using AT1 and AT2 receptor antagonists. As shown in Figure 3A, the AngII-mediated increase in proteoglycan synthesis was abolished by pretreatment with the AT1 receptor–specific antagonist, losartan, and unaffected by the AT2 receptor–specific antagonist, PD123319, demonstrating that the changes seen were mediated exclusively by the AT1 receptor. To further characterize the changes mediated by AngII, experiments were performed with protein kinase C (PKC) and tyrosine kinase inhibitors. As shown in Figures 3B and 3C, the AT1 receptor–mediated increase in proteoglycan synthesis

Table 1. Effects of AngII on glycosaminoglycan subtype synthesis in vascular smooth muscle cells

<table>
<thead>
<tr>
<th>Incorporation (cpm/μg protein)</th>
<th>AngII (−)</th>
<th>AngII (+)</th>
</tr>
</thead>
<tbody>
<tr>
<td>3H-glucosamine</td>
<td>70 ± 7</td>
<td>190 ± 8</td>
</tr>
<tr>
<td>35S-sulfate</td>
<td>64 ± 5</td>
<td>131 ± 12</td>
</tr>
</tbody>
</table>

* Results are mean ± SEM.

b $P < 0.05$.

c $P < 0.01$ versus AngII (−).

Figure 1. Time course (A) and dose dependency (B) of angiotensin II (AngII) type 1 (AT1) receptor–mediated increases in proteoglycan synthesis. Vascular smooth muscle cells (VSMC) were treated with AngII (10−7 mol/L) for various times (A) or various doses of AngII for 48 h (B), and then cell-associated and secreted proteoglycan synthesis were determined as described in Materials and Methods. Results shown are mean ± SEM (n = 4 per assay point). **$P < 0.05$ versus control. *$P < 0.05$ versus control.

Figure 2. Analysis of proteoglycans in media from control and AngII-treated VSMC by DEAE-Sephacel (Amersham-Pharmacia) ion-exchange chromatography.
was unaffected by PKC inhibitors, chelerythrine and staurosporine, but completely inhibited by tyrosine kinase inhibitors, herbimycin A and genistein, suggesting that the changes were mediated by PKC-independent tyrosine kinase pathways. Interestingly, the effects of AngII were also attenuated by the mitogen-activated protein kinase (MAPK) kinase (MEK) inhibitor, PD98059, before stimulation with AngII (10\(^{-7}\) mol/L) for 48 h. Secreted proteoglycan synthesis was then determined as described in Materials Methods. Results shown are mean ± SEM (n = 4 per assay point). *P < 0.05 versus respective groups; **P < 0.01 versus respective groups.

Effects of AT2 Receptor Stimulation on Proteoglycan Synthesis and Proteoglycan Core Protein mRNA in VSMC-AT2

A different approach was used to examine the effects of AT2 receptor stimulation on proteoglycan synthesis. First, VSMC were transfected with the AT2 receptor using retroviral vectors as reported previously (10). The levels of AT2 receptors were increased from 0 to 280 ± 48 fmol/mg protein after retroviral transfection, whereas levels of AT1 receptor were unchanged at 664 ± 64 fmol/mg protein. These transfected VSMC-AT2 cells were therefore used for the studies on the AT2 receptor. As shown in Figure 5A, treatment of these cells with the AT2 receptor agonist, CGP42112A, resulted in a small (1.3-fold) but significant increase in proteoglycan synthesis, which was inhibited by the AT2 receptor antagonist, PD123319. A similar effect was seen when AT2 receptors were stimulated by a combination of AngII + losartan (Figure 5B). The effects of AT2 receptor stimulation were unaffected by the tyrosine kinase inhibitor, herbimycin, or the EGFR inhibitor, AG1478 (Figure 5C). Moreover, a significant increase was also seen in the presence of the MEK inhibitor, PD98059 (data not shown). In contrast, the effects of CGP42112A were attenuated by pertussis toxin, suggesting the involvement of Gi/o-depen-

Figure 3. Effects of inhibitors on AT1 receptor–induced increases in proteoglycan synthesis. Cells were pretreated with (A) the AT1 receptor antagonist, losartan (10\(^{-6}\) mol/L), or the AT2 receptor antagonist, PD123319 (10\(^{-6}\) mol/L), (B) the PKC inhibitors, chelerythrine (chele; 1 \(\mu\)mol/L) and staurosporine (stauro; 100 \(\mu\)mol/L), (C) the tyrosine kinase inhibitors, herbimycin A (herb; 1 \(\mu\)mol/L) and genistein (geni; 100 \(\mu\)mol/L), or the epidermal growth factor receptor (EGFR) tyrosine kinase inhibitor, AG1478 (250 \(\mu\)mol/L), or (D) the MEK inhibitor, PD98059 (10 or 30 \(\mu\)mol/L), before stimulation with AngII (10\(^{-7}\) mol/L) for 48 h. Secreted proteoglycan synthesis was then determined as described in Materials Methods. Results shown are mean ± SEM (n = 4 per assay point). *P < 0.05 versus respective groups.

Figure 4. Effects of AngII on proteoglycan core protein mRNA in VSMC. VSMC were treated with AngII (10\(^{-7}\) mol/L) for the indicated times, and levels of versican (A), biglycan (B and D), and perlecan (C) mRNA were assayed by Northern blot analysis. (upper panels) representative image; (lower panels) results of laser densitometric quantitation. The concentration of PD98059 was 30 \(\mu\)mol/L. Results shown are mean ± SEM (n = 4 per assay point). *P < 0.05 versus control; **P < 0.01 versus control.
dent pathways in the observed effects (Figure 5D). Results of Northern blot assays are shown in Figure 6. Expression of the mRNA for perlecan appeared somewhat increased, but the results did not attain statistical significance (Figure 6).

**Description of a Rat Perlecan cDNA Sequence from VSMC**

Experiments were performed to confirm the identity of versican and perlecan cDNA sequences from mRNA expressed in these cells. mRNA was subjected to RT-PCR, and then subcloned into the vector pcDNA3.1His/topo before sequencing. Sequencing of the versican plasmid revealed 100% identity to the previously reported versican sequence (22). In contrast, the homology between the VSMC-derived perlecan domain I cDNA fragment to the previously reported cDNA from rat epithelial cells (21) was only 83% at the nucleotide level (87% at the amino acid level), whereas the homology to mouse perlecan (23) were 94% and 98% at the nucleotide and amino acid levels, respectively. To confirm that the results were not an artifact, experiments were repeated using rat mesangial cells. These sequences were the same, and are presented in Figure 7.

**Discussion**

The vasoactive peptide hormone AngII plays a central role in the control of BP, renal hemodynamics, and fluid homeostasis. In addition, AngII acts directly on the vasculature as a growth factor and thereby plays an important role in the development of vascular hypertrophy and fibrosis and, consequently, of the vascular thickening that is a hallmark of hypertensive and arteriosclerotic disease (24). These pleiotropic actions of AngII are mediated by AngII receptors located on target tissues. Currently, the structure of two types of receptors, the AT1 and AT2 receptors, have been determined by molecular cloning. Most of the classic actions of AngII, namely vasoconstriction, stimulation of aldosterone secretion, and increased renal tubular sodium reabsorption, have been shown to be mediated by the AT1 receptor. In contrast, AT2 receptors have been shown to be involved in vasodilatation and inhibition of natriuresis. AT2 receptors have also been shown to have antigrowth and apoptotic functions (25,13). These findings have suggested that the AT1 and AT2 receptor functions may be predominantly antagonistic to each other and, therefore, that the net effect of AngII stimulation may be determined by the balance between the actions of these two receptor subtypes. As to the distribution of these receptors in the kidney, studies from our and other laboratories using immunohistochemistry, in situ hybridization, and receptor binding studies have shown that both AT1 and
AT2 receptors are expressed in the blood vessels of adult kidneys in both rat and humans (26,25,27).

In this study, we tested the hypothesis that both AT1 and AT2 receptors may be involved in the control of proteoglycans by VSMC. We found that stimulation of VSMC resulted in an increase in proteoglycan synthesis, as has been reported previously (16). We have shown that our VSMC express exclusively AT1 receptors with no AT2 receptors detectable by either binding studies (15) or RT-PCR (10); therefore, these results could be assumed to result from AT1 receptor stimulation. To confirm this assumption, we pretreated cells with the AT1 receptor antagonist losartan and found that the effects of AngII in these cells were totally inhibited, consistent with an AT1 receptor-mediated mechanism. The actions of AngII on VSMC have been shown to act by both PKC-dependent and PKC-independent pathways (24). We found that the AT1 receptor–mediated enhancement of proteoglycan synthesis was completely inhibited by tyrosine kinase inhibitors, herbimycin A and genistein, whereas the PKC inhibitors, chelerythrine and staurosporine, were without effect. Moreover, the actions of AngII were attenuated by the EGFR tyrosine kinase inhibitor, AG1478, and the MEK inhibitor, PD98059, suggesting the involvement of EGFR-dependent and MAPK-dependent pathways as major mechanisms in the control of proteoglycan synthesis by the AT1 receptor. These results are consistent with the findings of Eguchi et al. (28) and Moriguchi et al. (29), who found that AngII-induced EGFR transactivation plays a central role in the activation of the Ras-Raf pathway, leading to MAPK activation and stimulation of vascular hypertrophy. Our results suggest that a similar mechanism is involved in the control of proteoglycan synthesis by the AT1 receptor.

We also examined the effects of AT1 receptor stimulation on mRNA expression of the major core proteins expressed in VSMC and found that AngII stimulation resulted in significant increases in versican, biglycan, and perlecan in these cells. The composition of the GAG side-chains for each of these core proteins are predominantly CSPG, CS/DSPG, and HSPG, respectively; therefore, these results are consistent with the results of our GAG characterization assay using the enzymes Chondroitinase ABC and AC and heparitinase as well as the results of the ion-exchange chromatography. Although we did not examine changes in levels of other enzymes affecting GAG side-chain composition, the fact that the changes in GAG synthesis paralleled the changes in core protein mRNA is consistent with the assumption that all three biochemical classes of proteoglycans are up-regulated by AngII treatment. This is important, because each of these proteoglycans has diverse functions in the kidney and vasculature. Thus, versican has been implicated in the control of cell attachment and proliferation. Moreover, versican is up-regulated in atherosclerotic lesions, suggesting a role for this proteoglycan in the development of vascular disease (5). Similarly the expression of the CS/DSPG biglycan has been shown to be increased in atherosclerosis (5) as well as in areas of mesangial sclerosis and interstitial fibrosis in the kidney (6,7). Perlecan has also been shown to possess both angiogenic and growth-promoting effects (2). The up-regulation of these proteoglycans by AngII may be one of the important mechanisms by which AngII contributes to the progression of vascular hypertrophy and renal sclerosis. Concerning perlecan, we found that the sequence of our probe had high homology (98%) to amino acids 26 to 190 of mouse perlecan (23) but was somewhat different from the sequence previously reported from rat glomerular epithelial cells (21). We are currently performing further studies to investigate these differences.

We next examined whether AT2 receptor stimulation had any effects on proteoglycan synthesis. Although intact renal blood vessels express both the AT1 and AT2 receptors (26,25,27), VSMC lose AT2 receptors during the cell culture process (10,29); therefore, it has been difficult to directly examine the actions of AT2 receptors in vitro. We have reported previously that it is possible to reintroduce the lost AT2 receptors to VSMC by retroviral transfection and thereby to directly analyze the actions of the AT2 receptor in VSMC (10).

Treatment of these VSMC-AT2 cells with the AT2 receptor–specific agonist, CGP42112A, resulted in different results from those seen after AT1 receptor stimulation. Thus we noted a smaller but significant increase in proteoglycan synthesis with similar changes in proteoglycan core protein synthesis that did not attain statistical significance. Although a large body of evidence suggests that AT2 receptor actions are predominantly antagonistic to the AT1 receptor, we and others have reported that this may not always be the case in the vasculature. Specifically, Levy et al. (30) reported that the AT2 receptor antagonist, PD123319, attenuated AngII-induced vascular hypertrophy and fibrosis, a finding that has been corroborated in vivo by Cao et al. (31) and in vitro by our group (10). Moreover Senbomatsu et al. (32) reported that cardiac hypertrophy induced by pressure overload was attenuated in AT2 receptor–deficient mice. These findings challenge the view that AT2 receptor stimulation acts solely to attenuate growth and induce apoptosis with beneficial consequences for the attenuation of tissue hypertrophy. This is of clinical importance because the increase in the use of AT1 receptor antagonists results in a feedback increase in the renin-angiotensin system and enhanced stimulation of unprotected AT2 receptors, the long-term consequences of which are still unclear (12). Concerning the mechanisms of the AT2 receptor–mediated effects, studies from our and other laboratories have shown that several of the actions of this receptor may be mediated by Gai/o proteins (13,14), and the results of this study suggest that Gai/o may also be involved in the regulation of proteoglycan synthesis by this receptor. Interestingly, we found that the inhibitors that suppressed the AT1-mediated effects were ineffective in suppressing the AT2-mediated effects, providing further support for the notion that the signal transduction mechanisms of the two receptors are quite different.

In summary, the results of this study suggest that multiple mechanisms are involved in the control of proteoglycan synthesis by AT1 and AT2 receptors. These findings are of direct clinical relevance in view of the recent increase in the use of angiotensin-converting enzyme inhibitors and AT1 receptor antagonists in the treatment of hypertension and renal disease, which results in marked changes in the stimulation of both AT1 and AT2 receptors in target tissues. Proteoglycans play an important role in the pathophysiology of both renal/vascular fibrosis and proteinuria; therefore, understanding of these mechanisms may be important for designing newer and better strategies for limiting the progression of vascular disease and renal failure.
Acknowledgments
This work was supported in part by grants from the Ministry of Education, Science, and Culture of Japan and a grant-in-aid from Keio University Medical Science Fund, Japan.

References